

Insect cells respiratory activity in bioreactor

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Abstract Specific respiration rate (Q_{O_2}) is a key parameter to understand cell metabolism and physiological state, providing useful information for process supervision and control. In this work, we cultivated different insect cells in a very controlled environment, being able to measure Q_{O_2} . *Spodoptera frugiperda* (Sf9) cells have been used through virus infection as host for foreign protein expression and bioinsecticide production. Transfected *Drosophila melanogaster* (S2) cells can be used to produce different proteins. The objective of this work is to investigate respiratory activity and oxygen transfer during the growth of different insect cells lines as *Spodoptera frugiperda* (Sf9), *Drosophila melanogaster* (S2) wild and transfected for the expression of GPV and EGFP. All experiments were performed in a well-controlled 1-L bioreactor, with SF900II serum free medium. *Spodoptera frugiperda* (Sf9) cells reached 10.7×10^6 cells/mL and maximum specific respiration rate ($Q_{O_2 \max}$) of 7.3×10^{-17} molO₂/cell s. *Drosophila melanogaster* (S2) cells achieved 51.2×10^6 cells/mL and $Q_{O_2 \max}$ of 3.1×10^{-18} molO₂/cell s. S2AcGPV (expressing with rabies virus glycoprotein) reached 24.9×10^6 cells/mL and $Q_{O_2 \max}$

of 1.7×10^{-17} molO₂/cell s, while S2MtEGFP (expressing green fluorescent protein) achieved 15.5×10^6 cells/mL and $Q_{O_2 \max} = 1.9 \times 10^{-17}$ molO₂/cell s. Relating to the Sf9, S2 cells reached higher maximum cell concentrations and lower specific respiration rate, which can be explained by its smaller size. These results presented useful information for scale-up and process control of insect cells.

Keywords Dissolved oxygen · Specific respiration rate · *Drosophila melanogaster* · S2 · *Spodoptera frugiperda* · Sf9 · Transfected cell lines

Introduction

Animals cells large-scale culture have been widely used by research laboratories and industries to obtain a great variety of products destined to the investigation, diagnoses, therapeutic use and also biological control in agriculture. A great effort is being expended to develop methodologies that can allow high density cell cultures that lead to the production of great amounts of several products. Thus, technologies for the preparation of viral products, such as antigens and vaccines in cellular cultures, as well as proteins with therapeutic and diagnostic purposes have being developed, in the last years, with great success. Different culture systems, mainly in

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suspension, have revealed great efficiency for the preparation of viral products. These systems are characterized for permitting the cells to grow freely in the culture medium, without the necessity of support for the growth kept by adjusted systems of agitation and in favorable environment.

During the last decade, insect cells such as *Spodoptera frugiperda* Sf9 cells have been extensively used for recombinant proteins and biopesticides production. Baculovirus based expression systems have become very popular for the production of vaccine components, and one of its advantages over expression systems is that proteins are normally expressed at very high levels (Sanderson et al. 1999; Moiscard and Corso 1981). *Drosophila melanogaster* Schneider 2 (S2) cells have become increasingly utilized over the past few years for the expression of heterologous proteins. High levels of protein expression with pharmacological and biotechnological interest can be achieved using *Drosophila* Expression System procedure (Invitrogen). After three weeks of selection, this system using *Drosophila* promoters was able to generate a stable polyclonal cell line processing up to 1,000 gene copies per cell. Proteins of prokaryotic, eukaryotic and viral origin have been expressed in this system showing to be appropriately processed and biologically active (Li et al. 1996; Delm et al. 1999; Perret et al. 2003; Chang et al. 2005).

Insect cell cultures are easier to handle than mammalian cells, being capable to multiply in monolayers or in suspension at temperatures ranging from 25 to 30 °C. They show, for instance, a good resistance to shear stress as well as to pH variations and as a consequence the culture procedures can be based on technologies currently used for animal cells, such as tissue culture flasks and spinners bottles and, in addition, can also be performed in orbital shaker bottles and on simpler media (Weiss and Vaughn 1986; Mitsuhashi 1998; Pereira et al. 2001; Weber et al. 2002).

The animal cells are strict aerobics, needing therefore constant oxygen supply. However, they have great sensitivity to stress caused by the oxygen transfer system, either due to the mechanical agitation system or the aeration system.

For the culture of these cells it is basic that the systems are well dimensioned in what refers to the culture medium, working temperatures, agitation and aeration conditions. Therefore are an efficient and

optimized oxygen transfer is necessary so that the system can be used in industrial scale.

The continuous transference of oxygen to the cellular culture is important, since the oxygen is consumed on the oxidation of the glucose, and however its solubility in water is low, becomes many times a limiting factor when scaling-up. Therefore, the study of the degree of respiration intensity (specific rate and total oxygen consumption) and of the optimization of the conditions of culture in biorreactor serve to guide other studies related to the use of insect cells for scale-up and process control.

Materials and methods

Cell lines

Spodoptera frugiperda (Sf9) cells (ATCC 1711) were grown and maintained in the serum free SF900II, wild S2 cells (Invitrogen obtained from isolated cells of a primary culture of *Drosophila melanogaster* embryos in later periods of development) with passage 65; S2 cells transfected with the vectors pAcGPV and pCoHygro (S2AcGPV) constructed by Yokomizo et al. (2007) that express constitutively glycoprotein G of the rabies virus; S2 cells transfected with the vector pMtEGFP (S2MtEGFP), in passage 77, constructed by Santos et al. (2007) that express protein EGFP after the induction with copper sulfate (700 µM). These strains were generated in the Viral Immunology Laboratory of the Instituto Butantan (Brazil).

During maintenance all the cells were kept in Schott bottles (100 mL of total volume and 10 mL of cell cultures) in rotating incubator, 100 rpm and 28 °C, and cultivated by 4 to 5 days passage to maintain the cells in exponential phase and high viability.

The measure of cellular density was accomplished using a Neubauer camera and the cells were counted in optical microscope. The differentiation among the viable cells and non-viable was done through the addition of Tripzan Blue with concentration of 0.3 M (Merck).

Reactor system

A Discovery 100 bioreactor (Inceltech, France) was used in all runs, with total volume of 2 liters and

working volume of 1 liter and M.R.U. control system. This system measures pH (Mettler Toledo) and controls agitation at 100 rpm and temperature at 28°C. The original and dissolved oxygen (DO) control system was substituted by one with two mass flow controllers (Brooks Instruments, Holland). The oxygen electrode was calibrated in water saturated with air at temperature of 28 °C. The biorreator was connected to a microcomputer, which through Bioac software (Inceltech, France) registers the data. Software still allows the remote programming of the M.R.U. through the computer.

The oxygen transfer was accomplished through silicone tubing (Silastic RX 50, Laboratory Tubing, Dow Corning Corporation, US) with internal diameter of 2 mm, external diameter of 3.2 mm and length of 8.30 m, with surface in contact with the culture medium of approximately 0.083 m².

Due to the characteristic of the oxygen transfer system used in this work, that has a fixed k_La (volumetric oxygen transfer coefficient), it was possible to calculate OUR (Oxygen Uptake Rate) by the liquid phase balance (LPB) using the strategy described by Kamen et al. (1996) in insect cells cultivation. It was used a system where a constant flow was maintained and, for the modulation of the oxygen control, the partial pressure of oxygen was varied through the change in the fraction of oxygen in the aeration gas, being this regulation obtained by the use of a pair of mass flow controller that dosed the

proportion among the gases O₂ and N₂. In this configuration the transfer varies with the oxygen partial pressure in the interior of the membrane, which is modulated by the total pressure in the membrane and by the fraction of oxygen in aeration gas. The oxygen fraction in aeration gas was controlled by action sent by MRU controller to mass flow controller. The DO controller acts to keep DO set-point. In the present work, with C maintained controlled ($dC/dt = 0$) the oxygen in the liquid phase balance can be given by:

$$\text{OUR} = k_La (C_s - C).$$

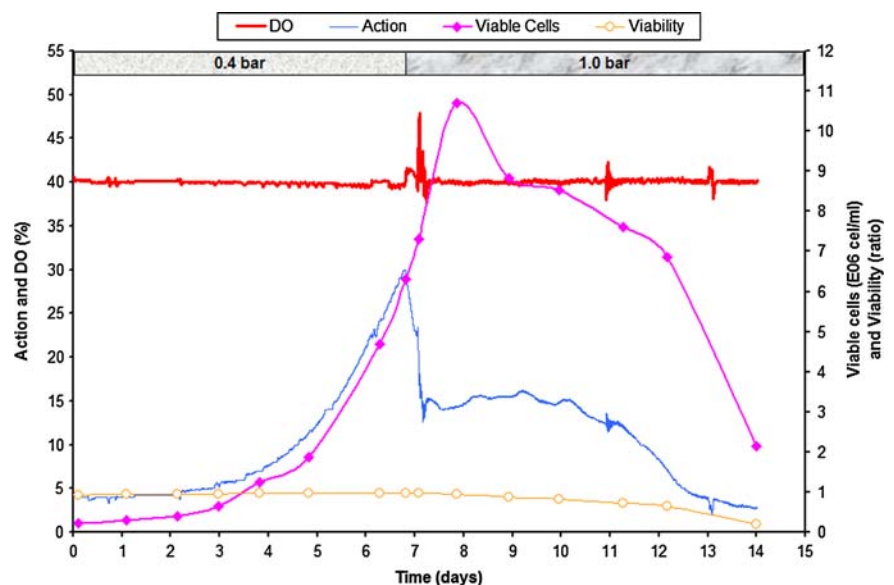
Considering constant k_La in the cultivation (calculated as $1.80 \pm 0.06 \text{ h}^{-1}$), knowing C (measured by the electrode) and C_s as a function to maintain set-point (Bailey and Ollis 1986), OUR can be calculated in real time, as well as the specific respiration rate (Q_{O_2}) that is given by:

$$Q_{O_2} = \frac{\text{OUR}}{X}.$$

Results and discussion

Spodoptera frugiperda (Sf9) cells in the passage 98 with no viral infection were cultivated in culture medium SF900 II at DO = 40% and the overpressure in the silicone membrane was maintained at 0.4 bar until day 7 and increased for 1 bar after then. That increase was

Fig. 1 DO, DO action controller, concentration of viable cells and viability cellular with the Sf9 cells in function of the time



necessary, because the oxygen supply was reaching its limit (maximum action of the mass flow controller) what could become a limiting factor. The agitation was maintained to 100 rpm, the temperature controlled at 28 °C. In Fig. 1, the main variables are observed. The agitation, temperature and dissolved oxygen were well controlled. The maximum DO controller action is 40% and was reached about day 7.

In this way, to avoid oxygen limitation problems, overpressure was increased for 1 bar and after small oscillation DO was again well controlled.

Starting from 0.22×10^6 cell/mL, the cells grew exponentially until a maximum cell concentration of

10.70×10^6 cell/mL approximately in day 8. The viability was about 97% until then declining thereafter. The maximum specific growth cell rate (μ_{\max}) was 0.70 day^{-1} verified till day 6 (Fig. 2).

In Fig. 2, it is observed OUR measurement and Q_{O_2} calculated through the liquid phase balance (LPB), where the maximum reached OUR was $1.43 \text{ mmolO}_2/\text{L h}$ verified during the exponential phase, while the maximum Q_{O_2} was $106 \times 10^{-18} \text{ molO}_2/\text{cell s}$ during the phase lag declining later for $74.4 \times 10^{-18} \text{ molO}_2/\text{cell s}$ during the exponential phase.

S2 wild cells in the passage 65 grew in SF900 II culture medium and at DO = 40% controlled with

Fig. 2 Measures of OUR and Q_{O_2} in liquid phase balance and specific growth cell rate with the Sf9 cells in function of the time

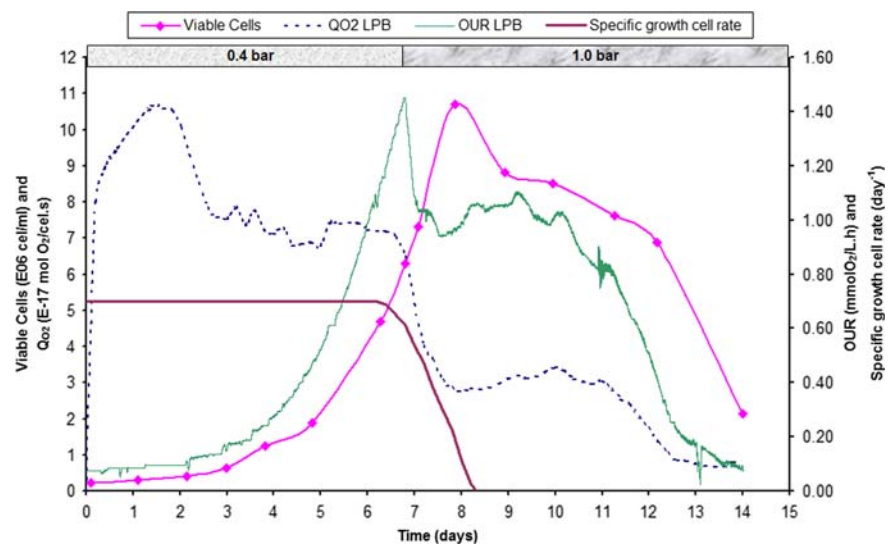
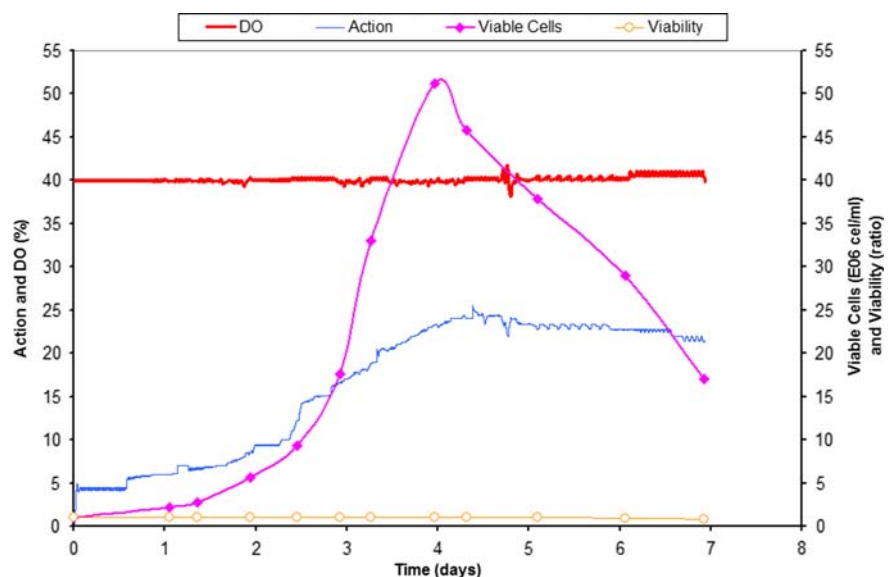


Fig. 3 DO, DO action controller, concentration of viable cells and viability cellular with the S2 cells in function of the time



overpressure in the silicone membrane of 0.5 bar (Fig. 3). The maximum requested action was about 24%, what shows that the maximum oxygen supply was limited to 30 mL/min. The initial cell concentration was of 0.98×10^6 cell/mL, reaching 51.20×10^6 cell/mL in day 4.

In Fig. 4, is observed that the maximum specific growth cell rate (μ_{\max}) was 1.07 day^{-1} verified in day 3.

OUR and Q_{O_2} were measured through the liquid phase balance (Fig. 4) OUR reached $0.98 \text{ mmolO}_2/\text{L h}$ verified after the exponential phase, while the maximum Q_{O_2} was $28.6 \times 10^{-18} \text{ molO}_2/\text{cell s}$

during the lag phase declining later till $7.42 \times 10^{-18} \text{ molO}_2/\text{cell s}$ during the exponential phase.

The run with the S2AcGPV cells had the same conditions of the former run (Fig. 5). The initial cell concentration was 0.50×10^6 cell/mL, reaching 26.6×10^6 cell/mL in day 8.

In Fig. 6, it is observed that the maximum specific growth cell rate (μ_{\max}) was 1.26 day^{-1} verified around the 5th day of cultivation.

OUR and Q_{O_2} were measured through the liquid phase balance (Fig. 6). OUR reached $0.87 \text{ mmolO}_2/\text{L h}$ verified in the end of the exponential phase, while the maximum Q_{O_2} was $58.4 \times 10^{-18} \text{ molO}_2/\text{cell s}$

Fig. 4 Measures of OUR and Q_{O_2} in the liquid phase balance and specific growth cell rate with the S2 cells in function of the time

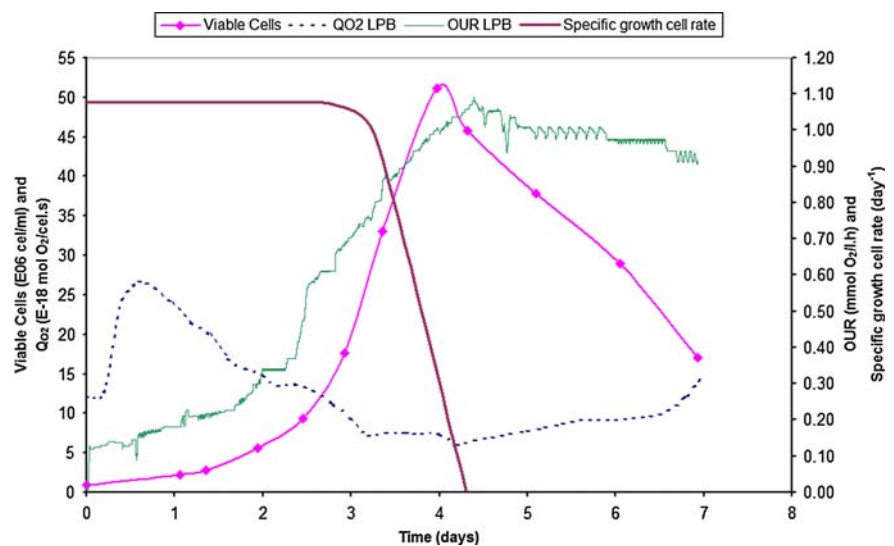
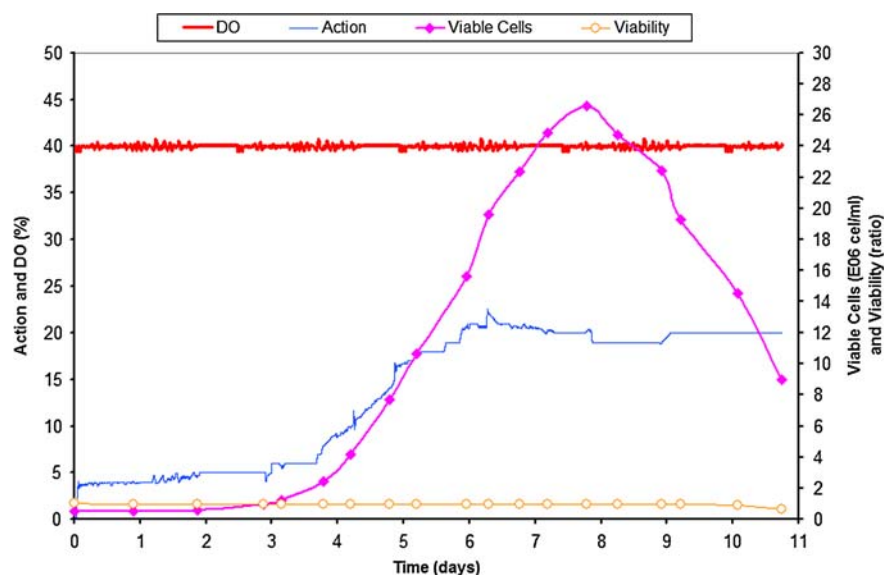


Fig. 5 DO, DO action controller, concentration of viable cells and viability cellular with the S2AcGPV cells in function of the time



during the lag phase declining later till 13.7×10^{-18} molO₂/cell s during the exponential phase.

The run with S2MtEGFP cell was carried through the same conditions (Fig. 7). The initial cellular concentration was 0.56×10^6 cell/mL, arriving in day 8 to 17.8×10^6 cell/mL.

In Fig. 8, it is observed that the maximum specific growth cell rate (μ_{\max}) was 0.68 day^{-1} verified in the moment of induction with copper sulfate, declining after that to $\mu_{\max} 0.38 \text{ day}^{-1}$.

OUR, $k_L a$ and Q_{O_2} were measured through the liquid phase balance (Fig. 8) OUR reached $0.69 \text{ mmolO}_2/\text{L h}$ verified in the end of the exponential phase, while Q_{O_2} reached 31.0×10^{-18} molO₂/cell s during the lag

phase declining later to 18.8×10^{-18} molO₂/cell s during the exponential phase.

Comparing the experiment of *Spodoptera frugiperda* Sf9 cell with the other runs with the *Drosophila melanogaster* S2 wild and transfected cells, it can be observed that Sf9 has a specific respiration rate larger than the others (Q_{O_2} around 74.4×10^{-18} molO₂/cell s). According to Kioukia et al. (1995) the specific rate of Sf9 is between 35 and 99×10^{-18} molO₂/cell s and according to Rodas et al. (2005) around 60.6×10^{-18} molO₂/cell s as shown in the Table 1.

The S2 wild cell line has a maximum specific respiration rate (during exponential phase) of

Fig. 6 Measures of OUR and Q_{O_2} in the liquid phase balance and specific growth cell rate with the S2AcGPV cells in function of the time

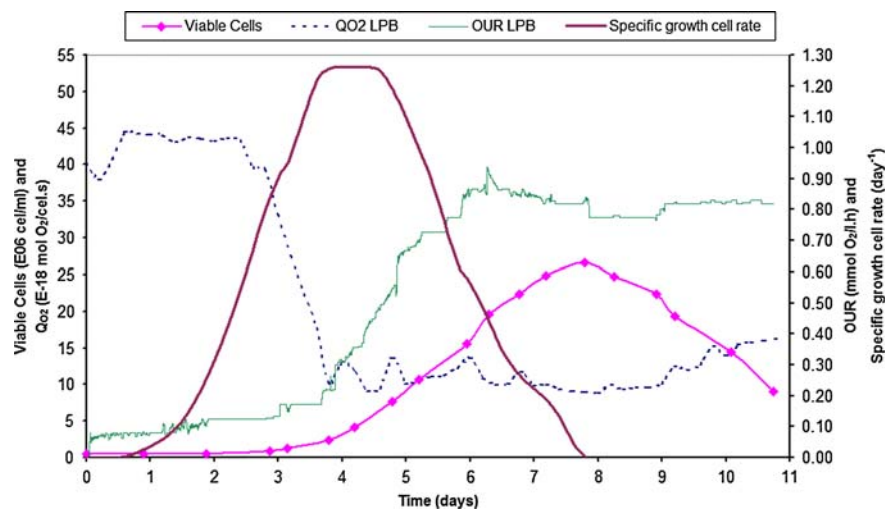


Fig. 7 DO, DO action controller, concentration of viable cells and viability cellular with the S2MtEGFP cells in function of the time

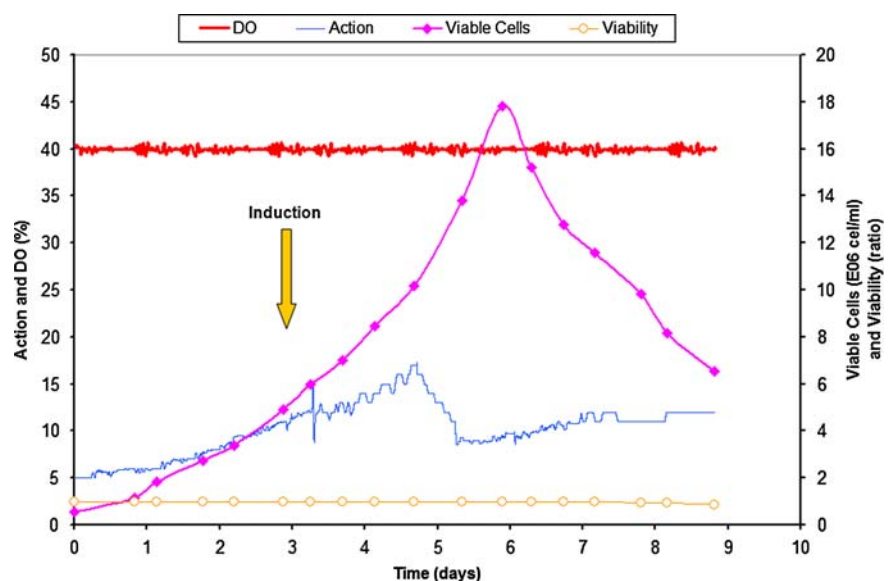


Fig. 8 Measures of OUR and Q_{O_2} in the liquid phase balance and specific growth cell rate with the S2MtEGFP cells in function of the time

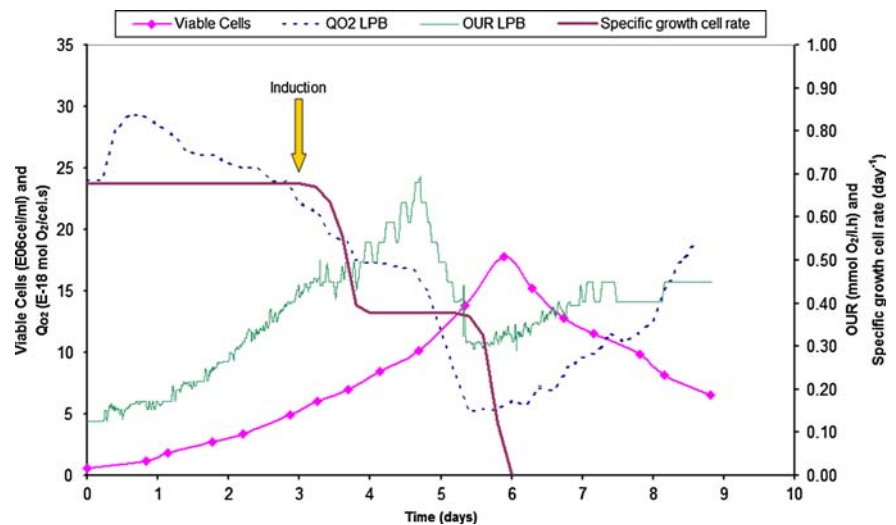


Table 1 Square comparative among runs with Sf9

	Data of Sf9 (this work)	Data of Sf9 (Rodas et al. 2005)
X_{initial} (cell/mL)	0.22×10^6	0.65×10^6
X_{max} (cell/mL)	10.70×10^6	8.80×10^6
OUR_{max} (mmolO ₂ /L h)	1.43	1.22
$Q_{O_2 \text{ max}}$ (molO ₂ /cell s)	74.4×10^{-18}	60.6×10^{-18}
μ_{max} (day ⁻¹)	0.70	0.65

Table 2 Maximum values found in the liquid phase balance (LPB)

Cell	X (cell/mL)	OUR_{max} (mmolO ₂ /L h)	Q_{O_2} exponential phase (10^{-18} molO ₂ /cell s)	μ_{max} (day ⁻¹)
Sf9	10.7×10^6	1.43	74.40	0.70
S2 p. 65	51.2×10^6	0.98	7.42	1.07
S2AcGPV	26.6×10^6	0.87	13.71	1.26
S2MtEGFP	17.80×10^6	0.69	18.84	0.38

15.18×10^{-18} molO₂/cell s and the transfected cell lines of 13.71×10^{-18} molO₂/cell s (S2AcGPV) and 18.84×10^{-18} molO₂/cell s (S2MtEGFP) (Table 2).

Through the present results, it can be seen that respiration activity of insect cells varies from cell line (Sf9 \times S2) and construction (wild and recombinant). These finding bring useful information for scale-up and control of insect cells bioprocess, specially considering that oxygen transfer may impose a strong limitation in bioreactor design.

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